Diabetes

Intracutaneous Transplantation of Islets within a Biodegradable Temporizing Matrix (BTM) as an Alternative Site for Islet Transplantation

Darling Rojas-Canales[1,8#], Stacey N. Walters[2,3#], Daniella Penko[1,8#], Daniele Cultrone[2], Jacqueline Bailey[2,3], Tatyana Chtanova[2,3], Jodie Nitschke[1,8], Julie Johnston[1,8], Svjetlana Kireta[1,8], Thomas Loudovaris[4], Thomas W Kay[4], Tim R. Kuchel[9], Wayne Hawthorne[5], Philip J. O'Connell[5], Greg Korbutt[6], John E. Greenwood[7]*, Shane T. Grey[2,3]*, Chris J. Drogemuller[1,8]* and P.Toby Coates[1,8]*

[1]Department of Medicine, University of Adelaide, Royal Adelaide Hospital Campus, Adelaide, South Australia.
[2]Transplantation Immunology Laboratory, Garvan Institute of Medical Research, Sydney, Australia.
[3]St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, Australia.
[4]St Vincent's Institute, Melbourne, Victoria, Australia,
[5]Westmead Hospital Sydney, New South Wales, Australia.
[6]University of Alberta, Canada.
[7]Burns Unit Royal Adelaide Hospital, Adelaide, South Australia, Australia.
[8]Central Northern Adelaide Renal and Transplantation Services (CNARTS) Royal Adelaide Hospital, Adelaide, South Australia, Australia.
[9]South Australian Health and Medical Research Institute (SAHMRI), Adelaide, South Australia, Australia.

Equal first co-authors
*Equal senior co-authors

Corresponding authors: STG (s.grey@garvan.org.au) +61 413 443 729; CJD (Chris.Drogemuller@sa.gov.au) +61 431 210 659; PTC (toby.coates@sa.gov.au) +61 439 901 856.

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Abstract

Intra-hepatic islet transplantation for type-1 diabetes is limited by the need for multiple infusions and poor islet viability post-transplantation. The development of alternative transplantation sites is necessary to improve islet survival, and facilitate monitoring and retrieval. We tested a clinically proven Biodegradable Temporizing Matrix (BTM), a polyurethane-based scaffold, to generate a well vascularized intracutaneous 'neo-dermis' within the skin for islet transplantation. In murine models, BTM did not impair syngeneic islet renal-subcapsular transplant viability or function, and facilitated diabetes cure for over 150 days. Further, BTM supported functional neonatal porcine islet transplants into RAG-1$^{-/-}$ mice for 400 days. Hence, BTM is non-toxic for islets. two-photon intravital imaging used to map vessel growth through time identified dense vascular networks, with significant collagen deposition and increases in vessel mass up to 30 days post-BTM implantation. In a pre-clinical porcine skin model, BTM implants created a highly-vascularized intracutaneous site by day 7 post-implantation. When syngeneic neonatal porcine islets were transplanted intracutaneously the islets remained differentiated as insulin producing cells, maintained normal islet architecture, secreted c-peptide, and survived for over 100 days. Here we show that BTM facilitates formation of an islet-supportive intracutaneous 'neo-dermis' in a porcine pre-clinical model, as an alternative islet transplant site.

Article Highlights

- Human and porcine pancreatic islets were transplanted into a fully vascularised Biodegradable Temporizing Matrix (Novosorb) that creates a unique intracutaneous site outside of the liver in a large animal pre-clinical model.
- The intracutaneous pre-vascularized site supported pancreatic islet survival for 3 months in syngeneic porcine transplant model.
- Pancreatic (human and porcine) islet survival and function was demonstrated in an intracutaneous site outside of the liver for the first time in a large animal pre-clinical model.

Introduction

Islet transplantation is an effective treatment for selected patients with type-1 diabetes (T1D), whereby allogeneic human islets, isolated from deceased organ donors, are infused via the portal vein, into the liver of T1D recipients with severe metabolic instability and hypoglycaemic unawareness [1-3]. One of the major limitations to wider application of this technology is the site of implantation, up to 60% of the transplanted islet mass is lost following infusion into the liver [4]. This transplant site requires access to the portal circulation by cannulation of the portal vein, either by direct venepuncture or by a trans-hepatic approach to embolize the islet cells into the hepatic vasculature. The hepatic microcirculation is characterized by low oxygen tension, high levels of endotoxin and active immunity which further compromises islet function [5,6]. Furthermore, islets are pro-thrombogenic cell clusters and therefore anticoagulation at the time of transplantation is required to reduce the potential for the 'Instant Blood-Mediated Inflammatory Reaction' (IBMIR). This increases the risk of anticoagulation-related complications [7]. Retrieval of transplanted cells is impossible and renders the potential use of newer alternative sources of insulin secreting tissues, such as stem cell-derived or xenogeneic islets, extremely hazardous [8]. Thus, the quest for alternative extra-hepatic transplant sites represents a significant target for the islet transplant field.

A variety of extra-hepatic alternative sites have been proposed including intra-muscular, intra-thymic, intra-medullary long bone marrow [9] and the subcutaneous site [10]. Of these proposed sites, the skin proffers the advantages of ease of accessibility, clinical monitoring and imaging, diagnostic biopsy and even excision of the grafted tissues. The skin has shown promise as an extra-hepatic alternative site in experimental rodent models [11], however, its major limitation is the relative hypoxic nature of the dermis and underlying fat [12]. The Biodegradable Temporising Matrix (BTM) is a wholly synthetic bilayer replacement scaffold,

developed for use in major burn injury [13-21]. The 'dermal' component is a 2mm-thick, open-cell polyurethane foam (marketed as NovoSorb®), designed to biodegrade predominantly by hydrolysis. A non-biodegradable polyurethane film (seal) is bonded to the upper surface and functions as a 'pseudo-epidermis', protecting the wound from the external environment, reduces evaporative water loss and minimizes wound contraction. After full integration of the dermal component, the seal bond is weakened and the superficial polyurethane film can be peeled away to expose the highly vascularized tissue bed below. Critical to the success of the BTM in wound management is the creation of a highly vascularized 'neo-dermal' intracutaneous space, which, unlike normal dermis, contains loosely folded and whorled collagen bundles, allowing 'space' for other cell types to be implanted [15,16]. In this study, utilising multiple *in vitro* and *in vivo* pre-clinical models, we describe the successful transplantation, survival and function of murine and porcine islets transplanted into the NovoSorb® polyurethane material, revealing the potential of an intracutaneous BTM site as a novel alternative site for islet transplantation.

Materials and Methods

NovoSorb™ Biodegradable Temporising Matrix (BTM with polyurethane film seal) and samples of NovoSorb® foam matrix lacking the superficial seal, were provided by PolyNovo Biomaterials Pty. Ltd. (Port Melbourne, Victoria, Australia). The unsealed foam was used for all experiments where the matrix was transplanted internally (i.e. in all sub-kidney capsule transplant experiments in mice [22] and all *in vitro* culture assays), where the function of the surface seal was not required.

Glucose stimulated insulin secretion assay and in vitro islet viability

NovoSorb® foam 3mm disks pre-soaked with complete medium (RPMI, 10% FCS) to ensure saturation were transferred to 24 well plates and loaded with islets. One millilitre of complete media was gently added to wells and islets were incubated for 3 days at 37ºC and 5%CO_2. Islets were recovered from the NovoSorb® foam for analysis of viability and glucose stimulated insulin secretion assay.

Mouse transplant models

Mouse studies were approved by the Garvan Institute's Animal Ethics Committee (ARA 17_27). Male RAG-$1^{-/-}$ mice (6-10 weeks of age) were used for sub-kidney capsule experiments and diabetes induced with alloxan. Diabetes was determined as blood glucose ≥16 mM on 2 consecutive days measured by Free-Style Lite glucometer and Abbott Diabetes Care test strips following tail tipping. For the surgeries, as previously described [23, 24] a left flank surgical incision was made to access the kidney. Using a 21 gauge needle, an insertion point made in the kidney capsule allowed for insertion of 3 mm x 3 mm sized NovoSorb® foam, positioned under the capsule on the kidney parenchyma surface, followed by wound is closure. At the determined time post-placement a second surgery is performed to access the kidney.

The integrated NovoSorb® foam were readily visualised on the kidney surface. As described above, a second small incision were made in the kidney capsule, facilitating the islet bolus infusion using a Hamilton syringe onto the NovoSorb® foam . Pressure from the capsule on the foam holds the placed islets in position. The kidney is recessed and the wound is closed.

Two photon *In Vivo* Microscopy

For *in vivo* imaging studies on host- and islet-BTM interactions, murine model was utilised where male Lysozyme M Cre mice were crossed with male ROSA$^{mT/mG}$ mice (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J Jackson Laboratories). Whereby macrophages, polymorphonucleocytes and some dendritic cells express GFP, while blood and lymphatic vessels express membrane tdTomato. Analysis of NovoSorb® foam vascularisation utilised a A Zeiss 7MP two-photon microscope (Carl Zeiss) with a W Plan-Apochromat 20×/1.0 DIC (UV) Visible-IR water immersion objective.

Neonatal Islet Preparation

Porcine studies were conducted in Good Laboratory Practice-accredited animal facilities and approved by the local animal ethics committees (SAMHRI: SAM206). Westran Neonatal Islet Cell Clusters (NICC) and Large White X Landrace Neonatal Porcine Islets (NPI) were isolated from 2-7 day old pig pancreata by dissection and collagenase digestion using standard protocols (25,26). Briefly, piglets were anaesthetised using ketamine/xylazine (0.75mL ketamine and 0.25mL xylazine). Their pancreata were surgically harvested and subsequently finely dissected into 2mm size pieces in HBSS (Gibco). The fragments were digested with Collagenase type V (Sigma) for 12-14 minutes at 37^{o}C, before filtration through a sterile mesh and washing twice (300xg) in HBSS with 1% porcine serum. Following culture in 150mm x 20mm Petri dishes, with Hams F-10 medium, at 37^{o}C and 5% CO_2) for 6 days, cells were

counted. Media changes were performed every 3 days. After culture, cells were counted relative to size to determine their Islet Equivalent Quantification (IEQ). Subsequently, these cells were transplanted into the intracutaneous site created following BTM implantation into adult pigs (weight 20-50kg).

Creation of an Intracutaneous Transplant Site using Biodegradable Temporizing Matrix (NovoSorb™ BTM)

BTM was transplanted onto the back flank of adult pigs as previously described [13]. For this study, either standard 2mm- or a specially customized 4mm-thick BTM was engrafted to create the intracutaneous islet transplantation site. In four wounds (40mm x 40mm) skin and subcutaneous tissue were removed to the level of the panniculus adiposus. This was performed under general anaesthesia induced by intramuscular ketamine (1mg/10kg) and maintained by 2.5% isoflurane via endotracheal intubation. The flank and back hair were clipped then shaved prior to wound creation. Four wound sites were prepared (two on each flank), equidistant (4 cm) from the spine, with 4 cm between ipsilateral flank wounds. All marked wound margins were infiltrated with 0.25% bupivacaine with 1:400,000 adrenaline, providing 12 hours of post-operative pain relief, and vascoconstriction minimising local blood loss. Pre-emptive fentanyl transdermal patch were applied to the ear 24 hours prior to surgery (delivering 2µg/Kg/hr), for improved control of post-operative pain . These were replaced at the time of surgery to provide a further 72 hours of post-operative pain relief. In addition, buprenorphine (Temgesic) 0.3-0.5mg/Kg was administered by intramuscular injection during anaesthetic recovery, and Carprofen 2mg/Kg available when required for breakthrough pain suspected by the husbandry staff in the research facility.

The wounds were dressed to reduce shear and minimise the risk of infection with Acticoat™ (Smith & Nephew Ltd, Hull, United Kingdom), held in place by Hypafix™ (BSN Medical

GmBH, Hamburg, Germany). A cotton wool/fabric "combine" dressing was applied before customised fabric jackets were fitted to protect the sites. Dressing changes were performed every 2-7 days for the duration of each experiment.

Pig Islet transplantation in large animals

Female Large White X Landrace pigs (Sus scrofa- initial weight 25-30kg) were used in allogeneic and xenogeneic transplant experiments, whilst female Westran (inbred) pigs were used for syngeneic islet transplants. Standard diet, *ad libitum* water and general animal care standard operating procedures were followed throughout the study. All animals were fasted overnight before surgery. Prophylactic antibiotic therapy consisted of intramuscular Co-Amoxyclav (Clavulox, 3.5 ml) post-surgery, and orally (Clavulox 250mg tablet, 2 tablets twice daily) throughout the post-operative transplant period. Those animals receiving allogeneic or xenogeneic transplants were placed on an immunosuppressive regime consisting of tacrolimus (Prograf→, Astellas 0.25mg/kg) and mycophenolate mofetil (CellCept →, Roche 500mg twice daily), which commenced 1-2 weeks prior to islet transplantation. Therapeutic drug monitoring (Roche Immunoassay) for tacrolimus was performed to determine drug levels.

BTM was integrated over 17-25 days, prior to islet transplantation. Islets were pelleted in polyethylene tubing (internal diameter 0.58mm and external diameter 0.965mm) or loaded into a catheter, prior to the removal of the non-biodegradable seal from each wound site (delamination). Using a 1mm steel rod, a channel was created to allow for the insertion of the islet loaded cannula. A Hamilton syringe was used to slowly deposit islets into the created channel. A split skin graft was taken from adjacent skin and placed over the wound at the completion of the islet transplant procedure (Figure 3C).

Syngeneic Islet Transplantation

NICC were isolated from Westran piglets by the isolation centre at Westmead Hospital [26], Sydney, New South Wales and transported to Adelaide, where they were either transplanted that day, or placed into culture overnight prior to transplantation the following day. Islets (7,000 – 15,000 IEQ total / intracutaneous site) were washed with saline and loaded into cannulae as described above before transplantation into Westran recipients (40-50kg). Saline alone was used as a transplant control. Animals were humanely killed at 101 days post-transplantation for the long-term survival studies.

Allogeneic Islet Transplantation

Large White X Landrace NPI were isolated and cultured as previously described [25] prior to transplantation (2,500 - 40,000 IEQ total / intracutaneous site) into immunosuppressed adult recipients (20 - 40kg). The NPI grafts were harvested following a 14-day engraftment period and assessed for survival and revascularisation.

Xenogeneic Islet Transplantation

Research-consented human islets (SAHMRI: SAM206) isolated at St Vincent's Institute Melbourne Victoria were transported to Adelaide, South Australia where they were cultured for a maximum of 6 days in CMRL complete medium supplemented with 2mM glutamax, 10mM HEPES, penicillin-streptomycin and 10% human albumin (Albumex 20, Red Cross of Australia) prior to transplantation as described above. Animals were humanely killed 7-28 days post-transplantation.

Immunofluorescence

All tissues were fixed with 10% buffered formalin for at least 3 days prior to processing. Samples were paraffin embedded and 5µm sections were processed for histological analysis.

Sections were deparaffinized and re-hydrated prior to heat-mediated antigen retrieval (Citrate buffer pH 6). Sections were incubated with Guinea pig anti-human–insulin/glucagon antibody (AB7842; Abcam, Cambridge, UK) overnight as a primary antibody alone at 2µg/ml at 4°C. Insulin staining was visualised with secondary goat anti-guinea pig conjugated to Rhodamine fluorochrome (Jackson Immuno Research, Pennsylvania, USA) diluted 1:200 and incubated at room temperature in the dark for 1h. Otherwise, sections were co-stained with cross-reactive rabbit anti-mouse CD31 as a primary antibody overnight at 4°C (1:50 dilution of Ab28364, Abcam Cambridge, UK). An HRP-conjugated goat anti-rabbit polyclonal (1:2000) was used as the secondary antibody, before undergoing signal amplification with Tyramide Signal Amplification Kit as per the manufacturer's instructions (Molecular probes, ThermoFisher).

Glucose Stimulation Insulin Secretion (GSIS) Assay

Islets were handpicked into basal Krebs buffer (3 mmol/L glucose) at 37°C. Groups of 10 islets were then transferred into fresh basal Krebs buffer (3 mmol/L glucose) at 37°C for 1h, and the supernatant was collected. Islets were re-suspended in a 20 mmol/L glucose Krebs buffer (high) at 37°C for 1h. Porcine insulin was analysed using ALPCO Human Ultrasensitive Insulin Elisa Catalog Number: 80-INSHUU-E01.1 Human c-peptide was analysed using Roche Immunoassay (SA Pathology). For Intravenous Glucose Tolerance Testing (IVGTT) glucose was infused intravenously (0.5-g/kg of body weight) over one minute and blood glucose measurements taken immediately before infusion, at 30 minutes and 60 minutes post-infusion.

Vascular Density Quantification

For particle analysis of CD31 (Vascular Density) immunofluorescence staining, images were taken on a Zeiss confocal microscope at a 20X magnification. Tissue blocks were randomly selected and 6 representative images from each section (n=30) were analyzed using ImageJ

(with FIJI plugins) (National Institutes of Health, Bethesda, Maryland, USA) to determine CD31 positive pixels per total image area (%).

Data and resource sharing

All data generated or analyzed during this study will be made available in full upon request to the lead authors.

Results

Culture of islets in the presence of NovoSorb® foam does not affect viability or function

To assess the effect of BTM on islet viability and function *in vitro*, murine and human pancreatic islets were cultured within NovoSorb® foam for 72 hours. The islets were then extracted for analysis. Islet viability was determined using Annexin/Propodium iodide (PI) staining. No difference was observed with islets cultured in the presence of NovoSorb® foam (Figure 1A and 1B), and no deleterious effect on islet function, as measured by GSIS assay (Figure 1F).

Islet transplant into pre-engrafted NovoSorb® foam under the renal capsule supports long-term viability and function

Islet transplantation under the renal capsule is considered an optimal site for measuring engraftment and function, and therefore, suitable to test for potential negative effects of the NovoSorb® foam on islet engraftment and function *per se*. Accordingly, murine islets were transplanted under the kidney capsule of alloxan-induced diabetic immune deficient RAG-1$^{-/-}$ mice, with or without pre-engrafted NovoSorb® foam. Both control and treated mice showed prompt cure, with restoration of normal blood glucose to over 150 days (Figure 1G). Figure 1C shows the light microscopic appearance of the NovoSorb® foam under the kidney capsule with

insulin staining within the NovoSorb® foam containing islet graft (Figure 1D). Long term islet function within the NovoSorb® foam graft was then assessed using neonatal porcine islets (NPI) transplanted under the kidney capsule, with or without pre-engrafted NovoSorb® foam, into immune deficient RAG-1$^{-/-}$ mice. Serial blood samples were taken for over 400 days which showed increasing levels of porcine insulin, indicating no deleterious effect of the NovoSorb® foam on long-term islet maturation and function (Figure 1H). Macroscopic examination of the murine kidney-NovoSorb® foam graft (Figure 1E) shows visible vessels (black arrows) growing over the surface of the implanted foam.

Impact of NovoSorb® foam on neo-vascularisation

The pro-angiogenic characteristics of NovoSorb® foam post-transplantation under the kidney capsule were investigated by *in viv*o two-photon microscopy. Recipient mice engrafted with NovoSorb® foam were examined at days 7, 14 and 30. Collagen staining (blue) at the 3 time points (Figure 2A, 2D and 2G) indicates a progressive, incremental increase over the period studied. Vessel formation, as depicted by Evans Blue dye (white) reveals formation of immature microvessels by day 7 (Figure 2B), microvessel maturation at day 14 (Figure 2E) and further microvessel maturation along with formation of larger vessels at day 30 (Figure 2H; Online Supplemental Material 1 Video). The combined images in figures 2C, 2F and 2I illustrate both collagen and blood vessel development over time. Figure 2J shows the dense vascular development that occurred on the surface of the kidney immediately below the NovoSorb® foam at day 30, clearly highlighting the ability of the NovoSorb® scaffold to promote vascularization in this model. Collagen formation and vessel development is relatively constant at day 7 and day 14, however there is a significant increase in both collagen and vessel mass from day 14 to day 30, suggesting that transplantation of islets in this model is more likely to be optimal at the later timepoint (Figure 2K; Online Supplemental Material 1 Video).

In separate experiments, vascular development was assessed in both the syngeneic (Figure 3D - no immunosuppression), and the allogeneic (Figure 3E - with immunosuppression), porcine models. Vessel formation was evident by day 3 (allogeneic model) and day 7 (syngeneic model), and persisted through to the experimental endpoints of day 31 and day 118, respectively. Clear evidence of vascularity at day 118 indicates long-term persistence of the newly formed vascularity within the skin (Figure 3D – Day 118). Vascular development kinetics in the presence of immunosuppression was similar to that observed in the syngeneic model.

Intracutaneous Transplant of Porcine and Human Islets into pre-engrafted BTM

To test the intracutaneous site for islet transplantation, 3 large animal models were utilized:

1. Syngeneic intracutaneous NICC transplantation, Figure 3A (Westran NICC to adult Westran recipient without immune suppression) [23],

2. Allogeneic intracutaneous NPI transplantation, Figure 3B (Large White X Landrace NPI to adult Large White X Landrace recipient with immune suppression) [24] and,

3. Xenogeneic intracutaneous human islet transplantation, Figure 5G (Human cadaveric islet to Large White X Landrace pigs with immune suppression).

Syngeneic Porcine Islets long-term survival and integration with the vasculature created within the intracutaneous transplantation site

To evaluate the long-term viability of islets transplanted into the intracutaneous site without the need for immunosuppression, 15,000 NICC were transplanted into adult Westran recipients (syngeneic donor and recipient, Figure 3A). As Westran NICC mature over 90 days *in vivo*, grafts were left *in situ* for 101 days before retrieval. In the absence of immunosuppression,

intracutaneously transplanted Westran NICC were detected at 101 days after engraftment by the presence of insulin and glucagon positive cells (n=2; Figure 4F-I). Despite the low number of NICC initially transplanted, we were able to clearly identify their presence across multiple sections within the 40mm X 40mm X 2mm transplantation site. Insulin positive NICC can be seen adjacent to CD31-positive vasculature within the intracutaneous site at 101 days post-transplantation (Figure 4K).

Allogeneic Porcine Islets survive and integrate with the vasculature created within the intracutaneous site of immunosuppressed pigs

To assess the ability of islets to engraft into an intracutaneous site in a manner analogous to human islet transplantation, ABO blood group compatible allogeneic NPI (20,000 IEQ) from Large White X Landrace donor pigs were transplanted using the cannula technique (Figure 3C; image '5') into Large White X Landrace recipient pigs (Figure 3B). Recipient pigs received oral tacrolimus and mycophenylate mofetil immunosuppression, monitored by commercial immunoassay, and demonstrated comparable serum levels to conventional human intrahepatic islet allograft recipients (data not shown). Histological analysis of graft sites removed at days 2 (Figure 4A), and 14 post-transplantation (n=2; Figure 4B-E), readily identified allogeneic porcine islet allografts integrated within the intracutaneous site, with insulin- and glucagon-positive cells visible within the transplanted material. Interaction between the NPI and the intracutaneous site vasculature at day 14 is shown in Figure 4J, with insulin-positive NPI (red) immediately adjacent to CD31-positive vasculature (green).

Xenogeneic Human Islets transplanted into the intracutaneous site are viable and functional

To confirm that human islets were viable in the intracutaneous site, created following 24 days of BTM integration, human xenogeneic islets (62,500 IEQ) were transplanted into a Large White X Landrace adult pig treated with oral immunosuppression. The islet graft was harvested 7 days later (Figure 5G). Triple hormone positive human islets (Figure 5A and 5B) were clearly visible within the graft site, and dual staining with insulin and CD31 revealed integration of islets with the intracutaneous vasculature (Figure 5C-F). To examine human islet function in the intracutaneous site, 3 Large White X Landrace pigs were transplanted intracutaneously with human cadaveric islets under immunosuppression (oral tacrolimus/mycophenylate mofetil). Islet function was determined by measurement of human c-peptide out to 7 days post-transplantation. C-peptide was detected from random serum samples in all three pigs at multiple timepoints (Figure 5H) with levels ranging between 30-500 pmol/L.

Discussion

The skin as a potential site for beta cell replacement has been considered since 1894, when P. Watson Williams first unsuccessfully attempted to implant fragments of sheep pancreas into the skin of a patient with type-1 diabetes [27]. Although the skin is a hostile environment for cell transplantation, Pepper *et al* have utilized foreign body reaction induced by a 2cm nylon catheter to create a vascularized and fibrotic *subcutaneous* space, which was able to support islet cell implantation in rodent models [28, 29]. While islet function did not equal that observed for sub-renal capsule implantation [28], those pioneering rodent studies provide support for utilizing skin as a potential site for islet implantation. Here, we provide a novel approach to overcome the limitations of the skin as a transplantation site by creating a fully-artificial intracutaneous 'neo-dermal' site using a biodegradable polyurethane matrix. Until now, the skin has been a disappointing site for islet transplantation due to poor vasculature and the inability to maintain an appropriate microenvironment for islet survival and function. The clinical advantage of NovoSorb® BTM is its proven ability to promote functional vascularization of the implantation site, enabling transplanted islets not only to survive, but able to sample the blood delivered by afferent arterioles from the systemic circulation to detect serum glucose levels and subsequently deliver insulin into the efferent venular outflow channels back to the systemic circulation. This novel intracutaneous site can support mouse and porcine islet engraftment, survival and long-term function in mouse models of diabetes. In the large pre-clinical porcine model, intracutaneous islet transplants survived long-term with evidence of early function. Translation of intracutaneous islet transplantation to the clinic would require a BTM graft approximately 3cm x 5cm to accommodate the ≤ 3cc of purified islet tissue typically transplanted.

Generation and testing of islet encapsulation devices and related technologies are advancing through pre-clinical stages and are recognised as an engineering approach to overcome the need for heavy immunosuppression but also facilitate new sources of beta cells (8). Development of coatings and structures utilising synthetic PEG hydrogel macrodevice systems (32), conformal coating strategies (32), nanoporous polymer thread technology (33) as well as unique device structures (34) offer opportunities to advance durable beta cell replacement therapies (35). The intracutaneous islet transplant procedure used here differs from approaches designed to house islets internally and are surgically placed under the skin. These devices are typically not designed to biodegrade and can be seen as foreign to the body for the entirety they remain *in situ*. The BTM is completely biodegradable, such that once it has driven the vascular development and supported islet engraftment in the early post-transplant period it is completely degraded and excreted from the body. By 12 months post-transplant there is little to no BTM material remaining within the transplant site.In the generation of an intracutaneous 'neo-dermis' by using the BTM, an extensive vascular network of small arterioles, capillaries and venules is created within the dermal compartment, which subsequently is capable of fully supporting a split skin graft. Using this technique, severely burn-injured individuals have been able to survive previously catastrophic burn injury [36-38]. Additionally, the synthetic nature of the matrix does not provide a culture medium for organisms allowing its use to be extended to the management of wounds following severe cutaneous/subcutaneous infection (eg necrotising fasciitis) [39] and non-burn trauma [21]. During the development of the polyurethane material the formation of a highly vascularized dermal layer was observed, with loose, whorling bundles of collagen, allowing skin grafts placed on top of the newly-created dermal layer to survive. We hypothesized that this rich vascular bed, and the space afforded by loose collagen and oedema in the intracutaneous 'neo-dermis', might adequately support islet viability and function.

The BTM studied here is a fully biodegradable polyurethane matrix, which underwent 14 years of pre-clinical and clinical development culminating in regulatory approval in several jurisdictions, including 510k US FDA approval and use of the material in wounds in the US [13-18]. The product utilizes a non-degradable, polurethane removable seal, which prevents evaporative water loss from the wound, inhibiting the signal for fibrosis. Once the dermal foam component is fully integrated, the seal delaminates easily by gentle teasing. The superficial aspect of the underlying polymer separates to the level of the bond, allowing exposed polymer on the superficial surface of the 'neo-dermis' to retract back into the newly-created tissue. This ensures that all of the seal, and the bond, are removed during delamination, leaving a refreshed wound bed. The NovoSorb™ biodegradable polyurethane has been designed to maintain physical strength and structure for 3 months post-application. After this time point, progressive hydrolysis of the material results in matrix degradation into products which are easily excreted (CO_2, water) or utilized (lysine). Larger molecular residue is absorbed by macrophages/giant cells, leaving an intact blood vasculature, which has shown persistence in human biopsies out to 5 years. The huge majority of this process is completed by 12 months, certainly by 18 months there is no histological evidence of its implantation.

There are several strengths to this study which include the demonstration of long term islet survival in a robust syngeneic large animal model and islet survival in the background of systemic immunosuppression in both the allogeneic and xenogeneic porcine models with evidence of early function, as demonstrated by the detection of serum c-peptide, in the xenogeneic transplant group. Thus, supporting further translational development towards curative models. Another major strength of the study is that the porcine model represents the animal skin model most similar to humans. Unlike rodents and non-human primates, pigs lack

a panniculus carnosus (subcutaneous muscle layer under hair bearing skin), and instead possess a panniculus adiposus, a structure in common with human skin in all but a few locations. However, pig models also pose significant challenges (maintaining immune suppression, husbandry issues, dietary considerations), which make curative studies challenging. Nonetheless, given the physiological and architectural similarity of porcine and human skin, the current data would be supportive of future work using BTM to facilitate a novel alternative site for islet transplantation.

A current limitation of the study relates to the distribution and delivery of islets into the intracutaneous site. It is recognised that islets need to be within 200 micrometres of a blood supply for viability, hence the cannula route of administration used in this study, whilst appropriate for demonstration of proof of principle and function on implanted islets, is not yet optimised to deliver an islet mass necessary for insulin independence in large animals. Since the external diameter of the cannula was 500 micrometres, islets in the central core were potentially rendered ischaemic. Further experimentation to optimally deliver islets in proximity to vasculature is required. In addition, the dose of islets transplanted in the pre-clinical porcine models were not intended to be curative, only the xeno-islet transplant group were rendered diabetic to look for early evidence of function, and islet dose for all 3 models was governed by the islet isolation yield achieved.

Nevertheless, these studies show proof of principle in large animal models, that pancreatic islets may be successfully transplanted into an intracutaneous site mirroring human skin. Further characterization of this site with regard to potential reduction in IBMIR should also be performed. The advantage of being able to visualize the islet graft, biopsy or completely remove the cellular transplant opens this site as a safe option for transplantation of stem cell

and xenogeneic sources of insulin secreting tissue, with the potential for translation into clinical practice via this alternative transplant site.

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P. Toby Coates is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

P. Toby Coates and John E. Greenwood are stakeholders in Beta Cell Technology (BCT), a research and development company.

John E. Greenwood is the inventor of the NovoSorb® BTM and remains a shareholder in PolyNovo Biomaterials Pty Ltd (the manufacturers of NovoSorb® BTM).

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References

1. O'Connell, P.J., et al., *Multicenter Australian trial of islet transplantation: improving accessibility and outcomes.* Am J Transplant., 2013. **13**(7): p. 1850-8.
2. Hering, B.J., et al., *Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia.* Diabetes Care, 2016. **39**(7): p. 1230-40.
3. Marathe, C.,et al. , *Islet cell transplantation in Australia: screening, remote transplantation, and incretin hormone secretion in insulin independent patients.* Horm Metab Res. , 2015. **47**(1): p. 16-23.
4. Biarnés, M., et al., *β-Cell Death and Mass in Syngeneically Transplanted Islets Exposed to Short- and Long-Term Hyperglycemia.* Diabetes 2002, **51** (1) 66-72.
5. Cowley, M.J., et al., *Human islets express a marked proinflammatory molecular signature prior to transplantation.* Cell Transplant. 2012;**21**(9):2063-78.
6. Cantley J., et al., *A preexistent hypoxic gene signature predicts impaired islet graft function and glucose homeostasis.* Cell Transplant. 2013;**22(**11):2147-59.
7. Johansson, H., et al., *Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation.* Diabetes, 2005. **54**(6): p. 1755-62.
8. Odorico J., et al., *Report of the Key Opinion Leaders Meeting on Stem Cell-derived Beta Cells. Transplantation.* 2018 Aug;**102**(8):1223-1229.
9. Cantarelli, E., et al., *Bone marrow as an alternative site for islet transplantation.* Blood, 2009. **114**(20): p. 4566-74.
10. Anazawa, T., et al., *Current state and future evolution of pancreatic islet transplantation.* Ann Gastroenterol Surg., 2019. **3**(1): p. 34-42.
11. Pepper A.R., et al., *A prevascularized subcutaneous device-less site for islet and cellular transplantation.* Nat Biotechnol. 2015 May;**33**(5):518-23.
12. Evans, N.T. and P.F. Naylor, *The systemic oxygen supply to the surface of human skin.* Respir Physiol, 1967. **3**(1): p. 21-37.
13. Li, A., et al., *Evaluation of a novel biodegradable polymer for the generation of a dermal matrix.* Journal of burn care & research : official publication of the American Burn Association, 2009. **30**(4): p. 717.
14. Greenwood, J.E., et al., *Evaluation of NovoSorb Novel Biodegradable Polymer for the Generation of a Dermal Matrix Part 2: In-vivo Studies.* Wound Practice & Research: Journal of the Australian Wound Management Association, 2010. **18**(1): p. 24, 26, 28, 30, 32-34.
15. Greenwood, J.E. and B.L. Dearman, *Split skin graft application over an integrating, biodegradable temporizing polymer matrix: immediate and delayed.* J Burn Care Res, 2012. **33**(1): p. 7-19.
16. Dearman, B.L., et al., *"Take" of a polymer-based autologous cultured composite "skin" on an integrated temporizing dermal matrix: proof of concept.* J Burn Care Res, 2013. **34**(1): p. 151-60.
17. Wagstaff, M.J.D., et al., *A biodegradable polyurethane dermal matrix in reconstruction of free flap donor sites: a pilot study.* Eplasty, 2015. **15**: p. e13.
18. Wagstaff, M.J.D., et al., *Free Flap Donor Site Reconstruction: A Prospective Case Series Using an Optimized Polyurethane Biodegradable Temporizing Matrix.* Eplasty, 2015. **15**: p. e27.
19. Greenwood, J.E., et al., *Reconstruction of Extensive Calvarial Exposure After Major Burn Injury in 2 Stages Using a Biodegradable Polyurethane Matrix.* Eplasty, 2016. **16**: p. e17.

20. Wagstaff, M.J., et al., Greenwood, *Reconstruction of an Anterior Cervical Necrotizing Fasciitis Defect Using a Biodegradable Polyurethane Dermal Substitute.* Eplasty, 2017. **17**: p. e3.
21. Damkat-Thomas, L., et al., *A Synthetic Biodegradable Temporising Matrix in Degloving Lower Extremity Trauma Reconstruction: A Case Report.* Plastic and Reconstructive Surgery, Global Open, 2019. 7(4): p. e2110.
22. Grey S.T., et al., *Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function.* J Immunol. 2003;**170**(12):6250-6.
23. Malle, E.K. et al *Nuclear factor κB -inducing kinase activation as a mechanism of pancreatic β cell failure in obesity* J Exp Med 2015 **212**(8):1239-54
24. Zammit, N.W. et al *Low-dose rapamycin unmasks the protective potential of targeting intragraft NF-kB for islet transplants* Cell Transplant 2013 22(12):2355-66
25. Jimenez-Vera, E., et al., *Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: in vivo and molecular profiles.* Xenotransplantation, 2015. **22**(2): p. 114-23.
26. Korbutt, G.S., et al., *Large scale isolation, growth, and function of porcine neonatal islet cells.* J Clin Invest, 1996. **97**(9): p. 2119-29.
27. O'Connell, P., et al., *Genetic and functional evaluation of the level of inbreeding of the westran pig: A herd with potential for use in xenotransplantation.* . Xenotransplantation, 2005. **12**(4): p. 308-15.
28. Stokes, R., et al., *Transplantation sites for porcine islets.* . Diabetalogia, 2017. **60**(10): p. 1961-1971.
29. Watson-Williams, P., *Notes on diabetes treated with extract and by grafts of sheep's pancreas.* Br Med J, 1894(2): p. 1303-1304.
30. Pepper, A.R., et al., *A prevascularized subcutaneous device-less site for islet and cellular transplantation.* Nat Biotechnol, 2015. **33**(5): p. 518-23.
31. Pepper, A.R., et al., *Diabetes Is Reversed in a Murine Model by Marginal Mass Syngeneic Islet Transplantation Using a Subcutaneous Cell Pouch Device.* Transplantation, 2015. **99**(11): p. 2294-300.
32. Weaver, J.D., et al *Design of a vascularised synthetic poly (ethylene glycol) macroencapsulation device for islet transplantation* Biomaterials 2018. **172**:54-65
33. An, D., et al *Designing a retrievable and scalable cell encapsulation device for potential treatment of type 1 diabetes* Proc Natl Acad Sci U S A 2018. **115**(2):E263-E272
34. Smink, A.M., et al *In vivo vascularisation and islet function in a microwell device for pancreatic islet transplantation* Biomed Mater. 2021. **16**(3). doi:10.1088/1748-605X/abf5ec
35. Brusko T.M., et al *Strategies for durable β cell replacement in type-1 diabetes* Science. 2021 **373**(6554):516-522
36. Greenwood, J.E., et al., *Reconstruction of Extensive Calvarial Exposure After Major Burn Injury in 2 Stages Using a Biodegradable Polyurethane Matrix.* Eplasty, 2016. **16**: p. e17.
37. Greenwood, J.E., *The evolution of acute burn care - retiring the split skin graft.* Ann R Coll Surg Engl, 2017. **99**(6): p. 432-438.
38. Greenwood, J.E., et al., *Experience with a synthetic bilayer Biodegradable Temporising Matrix in significant burn injury.* Burns Open, 2018. **2**(1): p. 17-34.
39. Marcus, J.D.W., et al., *Biodegradable Temporising Matrix (BTM) for the reconstruction of defects following serial debridement for necrotising fasciitis: A case series.* Burns Open, 2019. **3**(1): p. 12-30.
40. Greenwood, J.E., *Hybrid Biomaterials for Skin Tissue Engineering.* 2016: p. 185-210.

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Figure 2 Improved vascularization induced by NovoSorb® foam/BTM shown by real time *in vivo* 2 photon microscopy (mouse)

To assess the impact of NovoSorb® [IDT] on tissue remodelling *in viv*o through time transgenic mice (LysGFPtdTomTg) expressing GFP^+ myeloid cells and blood and lymphatic vessels expressing tdTomato red were used. NovoSorb® was transplanted under the kidney capsule of the transgenic mice and 2-photon microscopy performed at days 7, 14 and 30. Figures 2A (7 days), 2D (14 days) and 2G (30 days) demonstrate blue collagen formation under the kidney capsule, with increasing density of blue over time. Figures 2B (7 days), 2E (14 days) and 2H (30 days) demonstrate white blood vessel formation, with increasing vascular formation over time. Figures 2C (7 days), 2F (14 days) and 2I (30 days) illustrate the merged collagen density with vascular changes indicating synchronous formation of loose whorls of collagen and vasculature with time. Black voids in all images are the NovoSorb® polmer under the kidney capsule. Figure 2J shows a composite low power (x40) view of the formed vasculature under the kidney capsule at the interface of the kidney tissue and the NovoSorb® [IDT] foam, with vessels staining white. Figure 2K depicts quantitative analysis of collagen formation and vessel development through time (days 7, 14 and day 30) .

Figure 3 Large White X Landrace pre-clinical large animal model of intracutaneous islet transplantation and vascular development

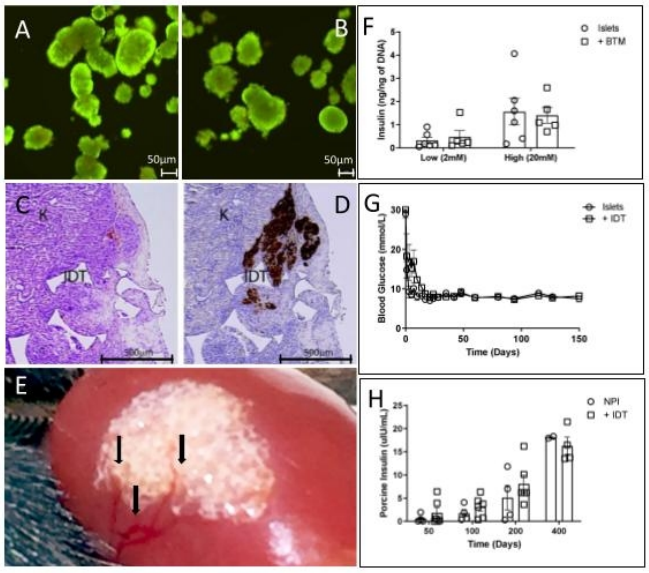
Westran pigs were engrafted with BTM followed by a syngeneic islet transplant of Neonatal Islet Cell Clusters (NICC) for 101 days prior to graft removal (Figure 3A). Immunosuppressed Large White X Landrace pigs were transplanted with allogeneic Neonatal Porcine Islets (NPI) following BTM engraftment with graft removal 14 days post-transplant (Figure 3B). Figure 3C depicts the key surgical timepoints of this pre-clinical porcine intracutaneous islet transplant model, beginning with marking out the BTM wound sites (Figure 3C-1 Day 0), wound creation and engraftment with four 40x40x2mm BTM foams (Figure 3C-2 Day 0), BTM graft vascularization (Figure 3C-3 Day 17-25), delamination of the seal at day 17 - 25 (Figure 3C-4), intracutaneous transplant of 15,000 NICC or NPI into one graft site in each pig (Figure 3C-5 Day 17-25), skin grafting of the transplant site (Figure 3C-6 Day 17-25), removal of the staples (Figure 3C-7 Day 31-39) and the transplant site 4 weeks after skin grafting (Figure 3C-8 Day 45-53). The remaining 3 sites of the Westran (Syngeneic) and Large White X Landrace (Allogeneic) pigs were serially biopsied to document the course of vascularization at the time points indicated, Figure 3D for the syngeneic model and figure 3E for the immunosuppressed allogeneic model, by staining with CD31 (green fluorescence) out to day 118 or day 31 post-BTM engraftment, respectively.

Figure 4 Normal islet hormone expression and viability following intracutaneous transplantation of syngeneic (NICC) islets for 101 days or Allogeneic (NPI) islets for 2 days and 14 days

BTM grafts were harvested at day 101 (NICC) or days 2 and 14 (NPI) and processed for insulin, glucagon and CD31 staining. Figure 4 shows the nuclear stain, DAPI (blue); porcine insulin-positive (red) and glucagon-positive (green) cells of NPI at 2 days post-transplantation (A) and 14 days post-transplantation in pig 1 (B, C) and pig 2 (D, E), and NICC 101 days post-transplantation into Westran pig 1 (F, G) and pig 2 (H, I). Figure 4J shows NPI on day 14 post-transplantation and Figure 4K, NICC on day 101 post-transplantation both showing the presence of insulin-positive (red) cells adjacent to CD31-positive (green) vasculature within the intracutaneous transplantation site.

Figure 5 Human pancreatic islets transplanted into the intracutaneous site in Large White X Landrace Pigs under immunosuppression are viable after 7 days

Figure 5G is a schematic of 62,500 IEQ human xenogeneic islets transplanted into an intracutaneous graft site in a Large White X Landrace pig following 24 days of BTM engraftment. Immunosuppression with tacrolimus and mycophenylate mofetil was initiated 16 days prior to islet transplant and the entire graft site was collected 7 days later. Triple islet hormone staining (Figure 5A & 5B) clearly identifies insulin- (red), glucagon- (green) and somatostatin-positive (purple) cells within the islet graft. Vascularized human islets in the intracutaneous site are depicted in Figures 5C and 5E, and the presence of intra-islet vasculature in high-powered images in Figures 5D and 5F, with insulin-positive cells (red) and CD31-positive vasculature (green). Figure 5H depicts the human c-peptide levels, from random serum samples, in 3 immune suppressed pigs following xenogeneic intracutaneous islet transplantation across multiple timepoints out to 7 days post-transplant.



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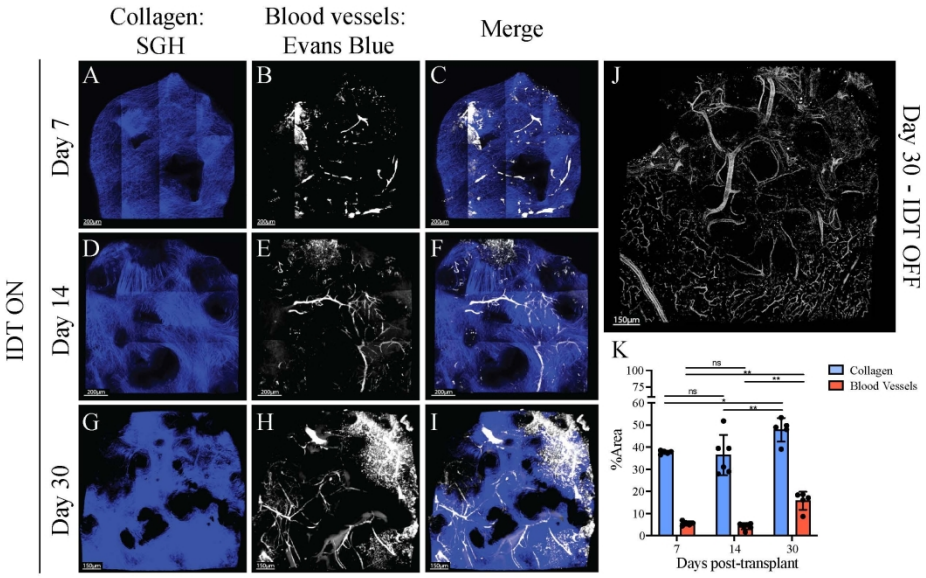

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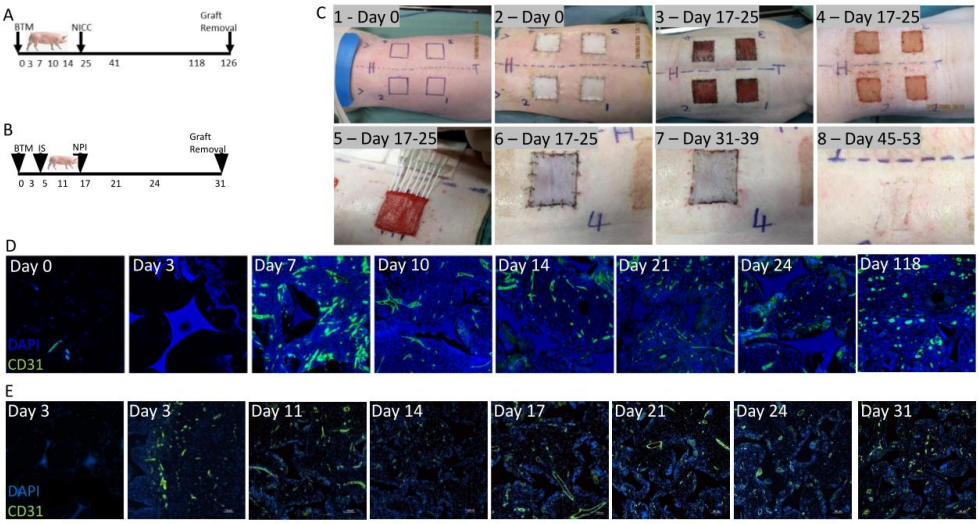

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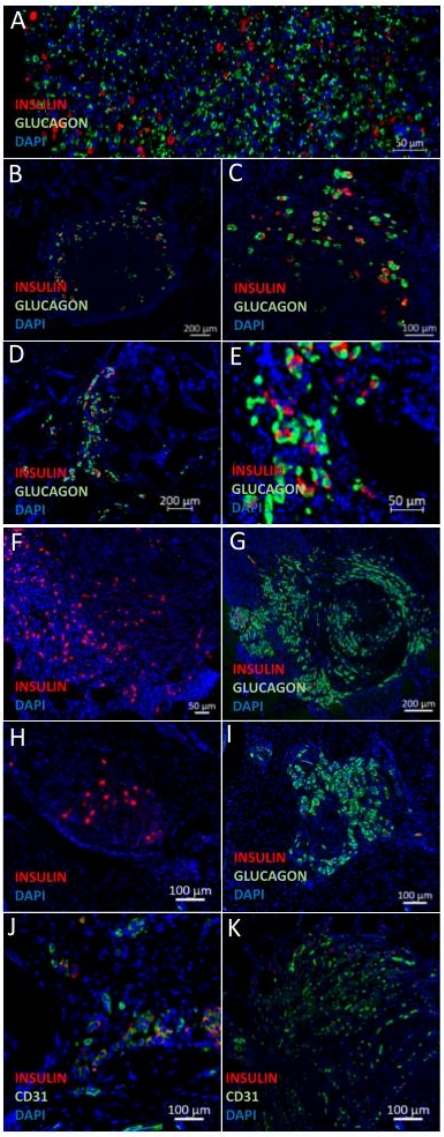

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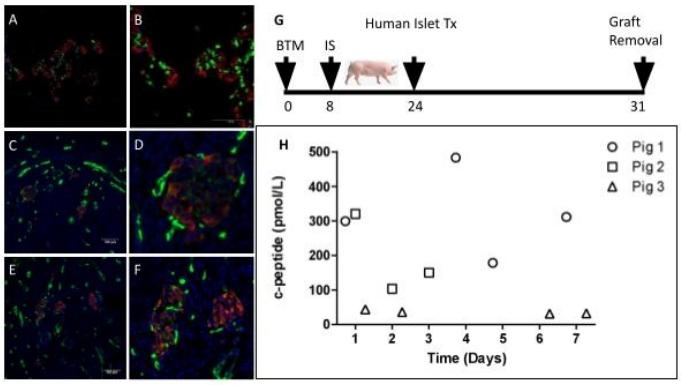

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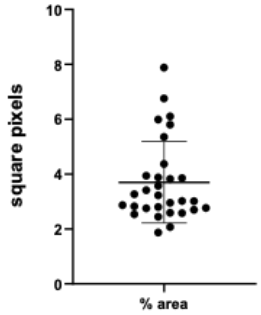
Online Supplemental Materials 1: Quantitative data on vascularization degree in BTM-islet transplants in porcine skin. Data represent analysis of CD31 staining area (ImageJ with FIJI plugins; National Institutes of Health, Bethesda, Maryland, USA) within islets placed into pre-implanted BTM ('neo-dermal' intracutaneous space) from two individual pig recipients at 64 days post implantation. Tissue blocks were randomly selected from BTM-islet site biopsies, sectioned and 6 representative images from each section (n = 30) were analyzed. Particle count (green channel = CD31 staining) was then analysed using the "analyse particles" option, which resulted in the total area for the white pixels per section. Vascular density was then calculated as a % of total tissue area. Methods are derived from: Schindelin J et al. "Fiji: an open-source platform for biological-image analysis", Nature Methods, pp. 676–82, 2012.



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25% Percentile	2.741
Median	3.121
75% Percentile	4.045
Maximum	7.879
Range	6.008
10% Percentile	2.453
90% Percentile	6.092
95% CI of median	
Actual confidence level	95.72%
Lower confidence limit	2.803
Upper confidence limit	3.852
Mean	3.700
Std. Deviation	1.486
Std. Error of Mean	0.2713
Lower 95% CI of mean	3.145
Upper 95% CI of mean	4.255
Coefficient of variation	40.16%
Geometric mean	3.463
Geometric SD factor	1.429
Lower 95% CI of geo. mean	3.031
Upper 95% CI of geo. mean	3.957
Harmonic mean	3.272
Lower 95% CI of harm. mean	2.922
Upper 95% CI of harm. mean	3.718
Quadratic mean	3.978
Lower 95% CI of quad. mean	3.258
Upper 95% CI of quad. mean	4.586
Skewness	1.339
Kurtosis	1.113
Sum	111.0

Online Supplemental Material

Intracutaneous Transplantation of Islets within a Biodegradable Temporizing Matrix (BTM) as an Alternative Site for Islet Transplantation

Darling Rojas-Canales[1,8#], Stacey N. Walters[2,3#], Daniella Penko[1,8#], Daniele Cultrone[2], Jacqueline Bailey[2,3], Tatyana Chtanova[2,3], Jodie Nitschke[1,8], Julie Johnston[1,8], Svjetlana Kireta[1,8], Thomas Loudovaris[4], Thomas W Kay[4], Tim R. Kuchel[9], Wayne Hawthorne[5], Philip J. O'Connell[5], Greg Korbutt[6], John E. Greenwood[7]*, Shane T. Grey[2,3]*, Chris J. Drogemuller[1,8]* and P.Toby Coates[1,8]*

[1]Department of Medicine, University of Adelaide, Royal Adelaide Hospital Campus, Adelaide, South Australia.
[2]Transplantation Immunology Laboratory, Garvan Institute of Medical Research, Sydney, Australia.
[3]St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, Australia.
[4]St Vincent's Institute, Melbourne, Victoria, Australia,
[5]Westmead Hospital Sydney, New South Wales, Australia.
[6]University of Alberta, Canada.
[7]Burns Unit Royal Adelaide Hospital, Adelaide, South Australia, Australia.
[8]Central Northern Adelaide Renal and Transplantation Services (CNARTS) Royal Adelaide Hospital, Adelaide, South Australia, Australia.
[9]South Australian Health and Medical Research Institute (SAHMRI), Adelaide, South Australia, Australia.

Equal first co-authors
*Equal senior co-authors

Corresponding authors: STG (s.grey@garvan.org.au) +61 413 443 729; CJD (Chris.Drogemuller@sa.gov.au) +61 431 210 659; PTC (toby.coates@sa.gov.au) +61 439 901 856.

Short title: Creating an islet-supportive intracutaneous 'neo-dermis'

Word count 4,692
Total Figures 5

Online supplemental material 1. 3-Dimensional Tile Map of IDT Interface with Blood Vessels. For this in vivo study a form of the Biodegradable Temporizing Matrix (Novosorb) was used that is identical except that it lacks the surface seal component of BTM and is referred to here as IDT. IDT was surgically placed under the renal capsule of C57BL/6 mice carrying a myeloid-GFP reporter and expressing tdTomatoe Red, and after 30 days the implantation site was imaged using 2-photon microscopy. Note the intense myeloid accumulation (Green fluorescence) at the IDT interface and the dense vascularisation (Evens Blue dye; appears white) evident in and around the transplant region. Blue areas represent collagen and are derived from the optical effect of second-harmonic generation. Red areas are non-myeloid cells and tissues constitutively expressing tdTomato fluorescence and include vessels. Angular 'dark' regions within the kidney tissue mass are the embedded structures of the IDT. See Methods for mouse line and 2-photon imaging technical information.